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THE FERTILIZATION-REACTION IN ECHINARACHNIUS PARMA.

IV. A Further Analysis of the Nature of Butyric Acid Activation.

E. E. JUST.

(From the Marine Biological Laboratory, Woods Hole, Mass., and the Zoölogical Laboratory, Howard University, Washington, D. C.)

Contents.

I. Introduction.

In a previous communication (Just, '19*c*) the writer has shown that the butyric acid activation of *Echinarachnius* eggs as shown by membrane lifting is complete since these eggs (following insemination) cannot be fertilized with sperm even after removal of the membranes. With membrane lifting, therefore, the eggs undergo an irreversible change. Those eggs, however, which following exposure to butyric acid at or below the optimum length of time for perfect membranes fail to show membranes are not activated because they are still capable of complete fertilization yielding on insemination normal membranes, cleavage, and larvæ. Likewise, eggs which have had an exposure to butyric acid be-

yond the optimum for membrane lifting, and which show thick jelly-like cortices without the wide perivitelline space so characteristic of both inseminated and butyric acid activated eggs, are still capable of fertilizaton although their development subsequent to insemination is abnormal. This response to insemination of these three classes of butyric treated eggs—activated, exposed but not activated, and over-exposed—constitutes a criterion in the analysis of activation as striking as the visible structural changes in the cortex of inseminated eggs which are the criterion of normal sperm activation. It seemed, with the aid of this criterion, desirable to attempt a further analysis of the activation process in *Echinarachnius,* and to this end the writer has made observations, herewith reported, on the duration of the fertilization capacity of butyric acid treated eggs. At the same time it was possible to study the rate of cytolysis of butyric acid treated eggs in sea-water as compared with normal eggs in sea-water. The results of these two lines of observations on butyric acid treated eggs give a possible explanation of the nature of butyric acid activation in *Echinarachnius* which involves the whole theory of activation. Following, therefore, the presentation of the observations on cytolysis (Part II.) and those on the duration of fertilization capacity (Part III.) is a general discussion of these results and their bearing on the theory of activation (Part IV.).

II. Observations on the Cytolysis of Butyric Acid Treated Eggs.

Now, it is a well-known fact that marine ova normally shed in sea-water when physiologically "ripe" for fertilization in sea-water cytolyze at a rate which varies with different species. Cytolysis of the uninseminated egg in normal sea-water is a natural phenomenon. Thus, according to Goldfarb:

"The consummation of the various deteriorating changes in ageing eggs is cytolysis and death.

"Cytolysis of sea-urchin eggs under the influence of various experimental conditions, such as saponin, salycyl, aldehydes, propyl alcohol, distilled water, etc., have been carefully described by Loeb. Loeb speaks of two methods of cytolysis, which he calls 'white' and 'black.'

"Ageing eggs cytolyze in the same two ways. In the 'white' or cytolysis by liquefaction, a changed permeability of the cortical layer permits an increasing volume of sea-water to enter the egg with a corresponding enlargement of the egg, a more viscous condition of the cytoplasm, a diminution in size of the protoplasmic granules, and an increasingly hyaline and translucent appearance of the egg. In the second type of cytolysis, there is either far less increase in size of the egg or no increase at all; the central mass remains opaque and becomes increasingly opaque; the cytoplasm is far less viscous; the outer surface which is hyaline is fragmented, and sometimes the inner mass as well, is fragmented, and the outer fragments fall off, with a consequent diminution in size of the eggs, even far below the normal.

"I was unable satisfactorily to establish whether these two are independent methods of cytolysis, possibly associated with different degrees of virility of the eggs or whether they are sequential phenomena. In most cultures, both types of cytolysis are seen at the same time.

"The onset differs in the eggs of different females, and, as in other evidences of ageing, this variation is due to differences in the physiologic condition of the eggs at liberation. Those eggs which were in good physiologic condition at liberation, cytolyzed late; those in poor condition, early. Eggs in relatively similar physiologic condition cytolyzed at a similar rate at the same temperature. The greater the temperature, the greater the rate.

"In *Toxopneustes,* cytolysis in any considerable numbers was first observed when ⅕ hour old in experiment 1, ⅕ hour old in experiment 3, 6 hours old in experiment 2 and 5, 11 hours old in experiment 4, 20 hours old in experiment 9. In *Hipponoe,* the rate of cytolysis is essentially the same as in *Toxopneustes.*

"In *Arbacia* it was much slower. Beginning, in the given experimental conditions, in about 28 hours as in experiment 17, and extending to 42 hours as in series 19, 46 hours in experiment 18, and later in other experiments. This difference in rate of cytolysis is in part due to differences in temperature of the sea-water in the two localities, but it is also due, and is another evidence of, a protoplasmic difference in the two species of eggs."

Now, the longevity of the egg in sea-water depends upon the

temperature, the physiological condition of the egg when shed—as determined by its size and presence of the jelly hull—the time in the breeding season, and the bulk of the eggs (that is, their concentration in a given volume of sea-water). To make comparisons, therefore, Goldfarb took precautions especially with reference to the last-mentioned factor, using as near as possible the same concentration of egg suspension. Nothing is clearer from these observations of his than the fact that eggs which are normally fertilized in sea-water after coming into sea-water and failing of insemination gradually die; for the cytolyzing egg is a moribund egg. Since it was not my intention to investigate the effect of various factors on this sea-water cytolysis—and Goldfarb's work made such an investigation unnecessary—I gave no particular attention to them in my observations. All that I wished to do in my study was to compare the rate of cytolysis in sea-water of eggs previously unexposed to butyric acid with those eggs in sea-water previously exposed for varying lengths of time to the action of this acid. My observations brought out a highly interesting fact; namely, that eggs under-exposed to butyric acid (*i.e.*, eggs that formed no membranes after transferral to normal sea-water) cytolyze at a slower rate than unexposed eggs. In other words, underexposure to butyric acid is beneficial to eggs in delaying the normal cytolytic effect of sea-water. To be sure, the factors temperature, seasonal variation, physiological condition of the ova, etc., play a part in the process; but, obviously, in any given observation in which a comparison is made between sea-water cytolysis and butyric acid cytolysis, since the eggs were from the same female and were approximately of equal mass in equal volume of water in the various dishes, the conditions were uniform.

Briefly, I found that if eggs of the sand dollar, *Echinarachnius parma,* after exposure to a mixture of *n*/10 butyric acid and sea-water (in the proportions 2 c.c. of acid plus 50 c.c. of sea-water) be transferred at varying intervals up to two or three minutes to dishes of pure clean sea-water they are cytolyzed during the ensuing thirty-six to forty-eight hours to complete disintegration. The rate of cytolysis—such factors as temperature, physiological condition of the eggs, seasonal variation, and mass of eggs to

the amount of sea-water used being taken into account—is determined by the length of the exposure to the butyric acid sea-water: (1) Eggs which have been exposed for one minute or more designated "over-exposed eggs" because they have remained in the butyric acid mixture beyond the length of time for best membranes cytolyze most rapidly; (2) eggs exposed under the optimum time for perfect membranes which are designated "under-exposed eggs" cytolyze more slowly; and (3) eggs which have had the optimum exposure (around 35 seconds) for perfect membranes cytolyze at a rate which is intermediate of the "over-" and "under-exposed" eggs.

A. The Method.

The procedure is as follows: Eggs shed into perfectly clean dry watch glasses or dry eggs washed off the ovaries as they exude or concentrated washed eggs are gathered up in a pipette. A tenth or twelfth of these eggs is set aside as a control in normal sea-water, the remainder exposed to 10 c.c. of butyric acid sea-water. From the butyric acid sea-water after 10, 15, 20, 25, 30, 35, 40, 50, 60, 90 and 120 seconds equal portions of the suspension are carried over to dishes containing 250 c.c. of sea-water. The control eggs are then removed to a dish of 250 c.c. of sea-water. Thus, a series of dishes containing approximately equal masses of eggs is made. Citations from the observations follow.

B. The Observations.

The following protocols, the first of the thirty 1919 observations and confirmatory of others previously made, are in no wise selected. They are given because they are simpler to present than some of the later which are bound up with other experiments of a different category—the presentation of which might draw attention from the salient feature which is under consideration.

Experiment 2 B. June 14, 8:00 A.M. Eggs taken from a very good female as they exude from the ovaries. Control in sea-water, remainder placed in 10 c.c. butyric acid sea-water from which portions removed to 250 c.c. of clean sea-water at intervals of 10, 15, 20, 25, 30, 35, 40, 50, 60, 90, and 120 seconds.

June 14, 3:00 P.M. Eggs examined and counts made for cytolyzing eggs with the following results:

	No.											
	1	2	3	4	5	6	7	8	9	10	11	12
Exposure to butyric acid in seconds	0	10	15	20	25	30	35	40	50	60	90	120
Per cent. of cytolyzing eggs	50	20	15	20	50	85	75	94	95	100	100	100

In this observation we find half the eggs in sea-water cytolyzed seven hours after removal from the female. Eggs treated with butyric acid for ten to twenty seconds fare better in sea-water; and eggs exposed for the optimum time while cytolyzing in high per cent. at this time still do not show complete cytolysis as do the over-exposed groups.

Experiment 6 B. June 15, 3:00 P.M. Eggs from one female shaken off ovaries into 10 c.c. of butyric acid sea-water; portions transferred to dishes each with 250 c.c. of sea-water after 15–120 seconds as shown below. Control in sea-water. Membranes best after the 35 and 40 seconds exposures, 93 and 90 per cent. respectively. A few perfect membranes after 60 seconds exposure, others contracted membranes, 90 and 120 seconds exposures give 100 per cent. contracted membranes.

June 16, 8:00 A.M. Dishes examined with the following counts shown:

	No.											
	1	2	3	4	5	6	7	8	9	10	11	12
Exposure to butyric acid in seconds	0	10	15	20	25	30	35	40	50	60	90	120
Per cent. of cytolyzing eggs	60	—	40	25	50	65	90	100	100	100	100	—

Seventeen hours after treatment with butyric a hundred per cent. cytolysis is shown in the over-exposed eggs. The under-exposed eggs cytolyze at a lesser rate than the control in sea-water.

Experiment 7 B. June 16, 7:00 P.M. Dry washed eggs from one female exposed to butyric acid up to 120 seconds (Nos. 2–11). Control (No. 1 in sea-water). Very few membranes after 35 and 40 seconds exposures. 60, 90, and 120 seconds exposures give 100 per cent. contracted membranes.

June 17, 8:00 A.M. Eggs from dishes show cytolysis as follows:

	No.											
	1	2	3	4	5	6	7	8	9	10	11	12
Exposure to butyric acid in seconds	0	10	15	20	25	30	35	40	50	60	90	120
Per cent. of cytolyzing eggs	10	—	24	36	41	90	94	100	99	100	100	100

This observation on washed eggs shows very clearly that over-exposed eggs thirteen hours after treatment are cytolyzed to a greater extent than under-exposed. The control eggs cytolyze more slowly than the under-exposed eggs which is an exception to the rule.

Experiment 8 B. June 17. Eggs from a very good female exposed to butyric acid sea-water from 15 to 120 seconds. Control in normal sea-water show some eggs devoid of jelly. Four hours later these eggs show per cent. cytolyzing as follows:

	No.											
	1	2	3	4	5	6	7	8	9	10	11	12
Exposure to butyric acid in seconds	0	10	15	20	25	30	35	40	50	60	90	120
Per cent. of cytolyzing eggs	50	—	29	21	74	80	86	85	50	100	100	—

Four hours after butyric acid treatment we find over-exposed eggs 100 per cent. cytolyzed. In most cases this percentage is reached after three hours. Cytolysis begins in these eggs within an hour after treatment when most of the other eggs including the control are intact. The beneficial effects of short exposure to butyric acid are strikingly brought out in this observation.

Experiment 9 B. June 19, 7:00 P.M. Eggs treated for 0, 15, 20, 25, 35, 40, 60, 90, and 120 seconds.

June 20, 8:00 A.M. These eggs after thirteen hours show the following:

	No.											
	1	2	3	4	5	6	7	8	9	10	11	12
Exposure to butyric acid in seconds	0	10	15	20	25	30	35	40	50	60	90	120
Per cent. of cytolyzing eggs	26	—	10	24	18	—	42	40	—	100	100	100

This observation demonstrates that while every egg of the over-exposed dishes is in cytolysis more than half the eggs of the 35 and 40 seconds exposures are intact and perfect in appearance. The large body of data universally shows that though cytolysis in over-exposed eggs may begin within an hour after exposure, the case is different for the eggs with membranes which are largely intact several hours after exposure. The membrane formation in these eggs is not therefore *per se* the cause of cytolysis. Moreover, here again as in other cases cited we note the beneficial effect of short exposure to butyric acid in arresting the normal cytolytic action of sea-water.

Experiment 10 B. June 24, 9:00 A.M. Eggs exposed to butyric acid sea-water from 15 to 120 seconds. Control in sea-water. At 3:30 P.M.—6½ hours after treatment—eggs examined for cytolysis. Another examination at 8:30 A.M., June 25. The counts of these two examinations are as follows:

	No.											
	1	2	3	4	5	6	7	8	9	10	11	12
Exposure to butyric acid in seconds	0	10	15	20	25	30	35	40	50	60	90	120
Per cent. of cytolyzing eggs after 6½ hours	24	—	2	12	36	50	76	30	—	60	80	100
Per cent. of cytolyzing eggs after 23½ hours	11	—	3	23	60	81	84	100	—	100	100	100

Comparison of the percentages of cytolyzing eggs six and one half hours and twenty-three and one half hours after treatment with butyric acid clearly shows that the cytolysis of eggs exposed for the optimum length of time is of a different order from that of over-exposed eggs. This was brought out repeatedly in the observations. Over-exposed eggs cytolyze rapidly *because* they are over-exposed; eggs with membranes cytolyze despite the fact that they have membranes.

Experiment 13 B. June 25, 11:20 A.M. Eggs from one female washed in 10 c.c. of sea-water twice in ten minutes. Sample removed to normal sea-water as control (No. 1); remaining eggs exposed to butyric acid sea-water for 15, 20, 25, 30, 35, 40, 60, 90, and 120 seconds.

June 26, 9:40 to 10:20 A.M. Eggs from each lot examined for cytolysis with the following counts:

	No.									
	1	2	3	4	5	6	7	8	9	10
Exposure to butyric acid in seconds.....	0	15	20	25	30	35	40	60	90	120
Per cent. of cytolyzing eggs.....	36	19	22	39	68	96	100	100	100	100

About twenty-three hours after treatment eggs of both the optimum (between 35 and 40 seconds exposure) and the over-exposed groups show high percentages of cytolysis. This result, however, should not be taken alone for as shown in other observations over-exposed eggs early show 100 per cent. cytolysis while optimum and under-exposed eggs are still intact.

If we tabulate the figures given in these seven protocols one may see at a glance that the shorter exposures to butyric acid are markedly beneficial in protecting the egg against the normal cytolytic action of sea-water.

TABLE I.

	No. of Dish.											
	(Control) 1	2	3	4	5	6	7	8	9	10	11	12
Exposure to butyric acid in seconds .	0	10	15	20	25	30	35	40	50	60	90	120
Per cent. of cytolysis 7 hours after.												
Ex. 2 B.....	50	20	15	20	50	85	75	94	95	100	100	100
Per cent. of cytolysis 17 hours after.												
Ex. 6 B.....	60	—	40	25	50	65	90	100	100	100	100	—
Per cent. of cytolysis 13 hours after.												
Ex. 7 B.....	10	—	24	36	41	90	94	100	99	100	100	100
Per cent. of cytolysis 4 hours after.												
Ex. 8 B.....	50	—	29	21	74	80	86	85	50	100	100	—
Per cent. of cytolysis 13 hours after.												
Ex. 9 B.....	26	—	10	24	18	—	42	40	—	100	100	100
Per cent. of cytolysis 6½ hours after.												
Ex. 10 B.....	24	—	2	12	36	50	76	30	—	60	80	100
Per cent. of cytolysis 23 hours after.												
Ex. 13 B.....	36	—	19	27	39	68	96	100	—	100	100	100

C. Discussion.

In the original observations the eggs were exposed to *one* c.c. of *n*/10 butyric acid plus fifty c.c. of sea-water for twenty seconds. Struck with the remarkable longevity of these eggs on re-

turn to normal sea-water as evidenced by their low per cent. of cytolysis and their retention of fertilization capacity as compared with the control in sea-water, the writer was led to continue the observations during succeeding summers using also the mixture *two* c.c. of *n*/10 butyric acid plus fifty c.c. of sea-water. Practically, if the egg does not form a membrane on removal from butyric acid, it gains through this acid treatment a resistance to the normal cytolytic action of the sea-water.

Examination of the tabulated data likewise clearly reveals that eggs exposed above one minute to the concentration of butyric acid employed are seriously injured. The individual observations as cited do not, however, so clearly show that these over-exposed eggs very early after removal from the butyric acid exhibit marked cytolytic changes. And for this reason: What primarily the writer aimed to determine was the point in time after removal from sea-water when one hundred per cent. of the *under-exposed* eggs (*i.e.*, eggs that show no membranes) completely disintegrate—a point by no means easy to fix because of the immunity against cytolysis conferred by the acid treatment. It may be recalled, nevertheless, that attention has been directed to the fact that cytolysis in the over-exposed egg begins very early. In general, over-exposed eggs showing one hundred per cent. thickened cortices give one hundred per cent. cytolysis within two hours after treatment. This fact was clearly revealed early in the study of the effect of butyric acid on these eggs. Another characteristic of over-exposed eggs as elsewhere noted (Just, '19*c*) is their response to shaking: the jelly-like cortex breaks and the eggs give off buds. That is, gentle shaking hastens cytolysis. In the egg with butyric acid membrane, the same or even more vigorous shaking produces merely a collapse of the membrane without injury to the vitellus. We must interpret these facts as meaning that the over-exposed egg is an egg in the initial stages of cytolysis the visible manifestation of which is the swollen cortex that so sharply differentiates it from the optimum exposed egg with its full round membrane and wide perivitelline space. But this interpretation by no means needs rest on the findings in *Echinarachnius* alone.

In the first place, Loeb has recorded similar observations for

speaking of *Arbacia* he says: "Since the membrane called forth by butyric acid is not always plainly visible, it is a prerequisite that always one set of such eggs should be set aside as controls to ascertain whether or not all the eggs disintegrate rapidly (if no second treatment is given them). Only if they all disintegrate rapidly have we any guarantee that in all of them the membrane formation has been effective" (Loeb, '15, page 262). Now, curiously enough, Loeb never saw membrane formation in *Arbacia* for he tells us of this egg: "When transferred to sea-water, they did not form a conspicuous fertilization membrane as did the eggs of *S. purpuratus* under the same circumstances, but only a fine gelatinous layer which was not easily visible" (Loeb, '13, page 71). Since, however, one can with proper exposure to butyric acid obtain very beautiful membranes in *Arbacia* and can obtain "the fine gelatinous layer" only through over-exposure it is clear that Loeb was dealing with over-exposed eggs which tend to disintegrate rapidly. Thus, according to Loeb over-exposure hastens cytolysis in *Arbacia.*

Again, Moore, working likewise with *Arbacia,* states: "Eggs exposed to butyric acid for slightly longer than the optimum time for membrane production do not produce membranes yet they cytolyze even more rapidly than in cases where membranes have been produced . . . they will many times have almost completely disintegrated when the first signs of cytolysis appear in eggs provided with membranes" (Moore, '16). Here, again, is noted a sharp difference between properly exposed and over-exposed *Arbacia* eggs.

Finally, Herlant ('17) working with *Paracentrotus* makes the point that it is not the formation of membranes that brings about cytolysis, for these eggs with butyric acid membranes may withstand the action of sea-water for twelve to fifteen hours before the onset of cytolysis. The egg cytolyzes despite the presence of the membrane because of internal changes due to nuclear activity.

In conclusion, then, we find that these facts taken together indicate that there is a clear cut difference between the butyric acid treated egg with a membrane and the over-exposed egg with a jelly-like cortex. The over-exposed egg is an egg in the initial stages of cytolysis, its jelly-like cortex marks the beginning of

cytolysis. But it is fallacious to argue that because the over-exposed egg is a cytolyzing egg the properly exposed egg, because of its membrane, is likewise a cytolyzing egg. That it cytolyzes is true, but it is also true that the normal uninseminated egg in normal sea-water cytolyzes. Instead, we might more logically contend that since under-exposed eggs are resistant to cytolysis that *membrane formation protects against cytolysis* for the under-exposed egg is no farther below the proper exposure for membrane production than the over-exposed egg is above it.

III. Observations on the Duration of Fertilization Capacity in Butyric Acid Treated Eggs.

In *Echinarachnius,* membrane elevation is a sign of complete activation whether the elevation is due to the agency of sperm or of proper butyric acid treatment for in either case the egg does not fertilize even after removal of the membrane. Activation is an irreversible reaction. But, neither the under-exposed egg that forms no membrane nor the over-exposed egg that likewise forms no membrane but exhibits a jelly-like cortex is activated since they are both capable of insemination; the former producing normal membrane, cleavage, and larva; the latter no membrane, abnormal cleavage, and larva. Thus, the response to insemination constitutes a physiological criterion of activation no less important than the striking morphological cortical changes. Since, now, we know that in *Echinarachnius* the effect of membrane production by butyric acid is the instant loss of fertilization capacity (Just, '19*c*) it would seem to be important in the analysis of the nature of butyric acid activation to know the duration of the fertilization capacity of under-exposed and over-exposed eggs. This section presents observations on this point which prove that, as in the case of the rate of cytolysis, there exist sharply defined differences among the three classes of eggs: under-, over-exposed, and activated.

A. The Method.

The method used in these observations is very simple. Following their exposure to butyric acid for varying lengths of time eggs are transferred to dishes of 250 c.c. of sea-water from

which samples are removed at intervals and inseminated with fresh sperm. Control eggs of bulk approximately equal to any of the exposed lots are similarly inseminated. To save time and material the eggs used for the study of cytolysis were frequently used for this set of observations. Every precaution was exercised to keep everything clean and sterile; likewise to procure conditions as uniform as possible.

B. The Observations.

The observations here presented are chosen for purposes of comparison from those that have already been reported in the section on cytolysis. And we may say at the outset that all these data show that the duration of fertilization capacity of the under- and the over-exposed eggs runs parallel with resistance to cytolysis. Under-exposure, then, not only protects against cytolysis but preserves the fertilization capacity; while over-exposure which hastens cytolysis likewise shortens the period after exposure during which the eggs will fertilize—their failure to fertilize constitutes the best index of initial cytolysis.

Experiment 2 B. June 14, 8:00 A.M. Eggs exposed to butyric acid for varying lengths of time then transferred to 250 c.c. of sea-water. Control in sea-water (see page 284).

12:02 P.M. Samples from dishes Nos. 1 (control) and 2–12 inclusive inseminated with fresh sperm suspension.

1:50 P.M. Cleavages in these dishes of inseminated eggs counted and percentages recorded as follows:

	No.											
	1	2	3	4	5	6	7	8	9	10	11	12
Exposure to butyric acid in seconds	0	10	15	20	25	30	35	40	50	60	90	120
Per cent. of cleavage	17	66	12	40	7	0	3	0	0	0	0	0

Here eggs inseminated about four hours after exposure show no cleavage in the over-exposed lots while the percentage of cleavage in the under-exposed lots is high—much higher in the case of the ten second exposure than in the control. Eggs with optimum treatment which form highest per cent. of membranes show no cleavage. There is a difference, however, between these

eggs with membranes and the over-exposed eggs; for, as previously shown (Just, '19*c*), eggs with membranes the instant of membrane elevation are incapable of fertilization; on the other hand, the over-exposed egg is fertilized on removal to sea-water. Its capacity for fertilization endures for one hour or more.

Experiment 6 B. June 16, 1:30 P.M. Eggs of Experiment 6 B whose percentages of cytolysis noted at 8:00 A.M. (see page 285) inseminated at 1:30 P.M. Cleavage counts at 4:30 P.M. follow:

	No.										
	1	2	3	4	5	6	7	8	9	10	11
Exposure to butyric acid.....	0	10	15	20	25	30	35	40	50	60	90
Per cent. of cleavage.....	0		75	15	10	3	1	0	0	0	0

Eggs we note that received a 15-second exposure show twenty-two and one half hours afterward 75 per cent. cleavages whereas normal eggs which have been in normal sea-water for the same length of time have lost completely their capacity for fertilization. This is by no means a solitary finding; the acid treatment markedly benefits the egg in protecting it against loss of fertilizing power. Activated eggs and over-exposed eggs have lost their capacity for fertilization.

Experiment 8 B. June 17, 8:35 A.M. 13 hours after exposure to butyric acid for 0, 15, 20, 25, 30, 35, 40, 50, 60, 120 seconds, eggs inseminated with fresh sperm suspension.

12:00 M. No cleavage in any dish.

These eggs lost their fertilization capacity relatively early.

Experiment 13 B. June 25, 5:50 P.M. Eggs from each of the dishes of the series exposed at 11:20 together with control (No. 1) inseminated with fresh sperm suspension.

7:30 P.M. Good cleavages with membranes up to and including No. 5. Best cleavages as to per cent., form, and size in Nos. 2, 3, and 4. Dishes set aside overnight.

June 26, 8:30 A.M. "Swimmers" counted in dishes, per cent. noted as follows:

	No.									
	1	2	3	4	5	6	7	8	9	10
Exposure to butyric acid in seconds	0	15	20	25	30	35	40	60	90	120
Per cent. of "swimmers"	31	76	62	71	38	0	0	0	0	0

This observation is cited to show that the stale egg previously treated with butyric acid is capable of developing into larvæ. No. 1 gave good larvæ; Nos. 2 and 3 gave normal larvæ; Nos. 4 and 5 were poor, large number of exogastrulæ.

July 1. Six determinations on different lots of eggs showed that eggs having 120 seconds exposure lost completely their fertilization capacity in 43, 61, 54, 73, 78, and 51 minutes respectively. Not a single egg had a membrane, but all had the jelly-like cortex. In each case, the 35 second exposure showed over 90 per cent. of eggs intact; the under-exposed eggs were all intact. The controls of the six lots showed 16, 19, 7, 14, 9, 3 per cent. cytolysis respectively.

C. Discussion.

The foregoing data constitute the evidence for the conclusion that short exposure to 2 c.c. of *n*/10 butyric acid plus 50 c.c. of sea-water prolongs the capacity of the egg to respond more or less normally to insemination. Compared with the normal egg in normal sea-water, the egg which treated with butyric acid fails to form a membrane not only is endowed with certain protection against the cytolytic action of sea-water but also is restrained from a too rapid loss of fertilizing power. This effect as in the case of cytolysis may be produced with one c.c. of *n*/10 butyric acid plus 50 c.c. of sea-water. Indeed, the consequence of the treatment with butyric acid may be an actual improvement of the normal egg as shown by the size, vigor, and longevity of the larva. Thus, eggs treated for ten seconds with the higher concentration of acid or for twenty seconds with the lower after twenty-four hours in sea-water show one hundred per cent. intact without any sign of cytolysis, whereas control eggs from the same female may show from fifteen to one hundred per cent. of cytolysis—observations frequently made during three seasons. Such eggs on insemination yield close to one hundred per cent.

fertilization but the control eggs may yield no fertilization. Freshly liberated eggs may be and often are inferior to these butyric acid treated eggs: they vary in size, in regularity of cleavage, and in vigor of larvæ.

The normally shed eggs of *Echinarachnius* allowed to stand in sea-water exhibit certain changes such as loss of jelly, change in size, etc., to complete cytolysis the beginning of which is the swelling of the cortex. If during the interval between shedding and complete disintegration—the time of which varies with temperature and with different lots of eggs—we inseminate these staling eggs periodically, we obtain additional evidence of physiological deterioration. For the uninseminated eggs of *Echinarachnius* the sea-water has an injurious action which manifests itself subsequent to insemination through the inability of the eggs to produce membranes, the loosening of blastomeres during cleavage, the production of aberrant larval forms, etc. (cf. Goldfarb's studies). Against this cytolytic effect of sea-water short treatment with butyric acid protects, the eggs cytolyzing at a slower rate and retaining fertilizing power for a longer period. It is only after several hours that these butyric acid treated eggs fail to respond to insemination.

Although the short exposure to butyric acid is thus beneficial in conserving fertilization power, longer exposures are decidedly harmful. An exposure of ninety seconds, for example, may be sufficient to bring about the complete loss of fertilizability within an hour. Such an exposure is beyond the optimum for activation and does not result in activation because the activated egg, which is the egg with full membrane, loses instantly with activation its capacity for fertilization. Over-exposure is comparable to prolonged staling in sea-water inasmuch as the effect of either is first to destroy the membrane, but the activable substances remain, albeit the long exposure has injured the egg, as evidenced through its jelly-like cortex, and the egg develops on insemination without membrane lifting. Failing of insemination the over-exposed egg soon loses its fertilization capacity because of the onset of death changes that manifest themselves in the thickening of the cortex which is the beginning of cytolysis.

In conclusion, these observations on the duration of the fer-

tilization capacity of butyric acid treated eggs show three physiologically distinct classes: under-exposed inactivated eggs whose fertilizability the acid treatment prolongs; activated eggs, with membranes, rendered incapable of fertilization at the instant of activation; and over-exposed inactivated eggs whose capacity for fertilization is cut short by the excessive action of the acid which initiates destructive changes.

IV. General Discussion.

1. The activation of the egg whether produced through sperm or butyric acid renders the egg incapable of insemination. Thus, Moore found *Arbacia* eggs following the successful production of membranes with butyric acid refractory to fertilization. In *Echinarachnius* (Just, '19*c*) subsequent to membrane formation with butyric acid the egg cannot be fertilized though the membrane be removed as soon as formed. Likewise, sperm activation renders the egg immune to the entry of any other sperm. The test, therefore, of complete activation is the response to insemination.

2. In *Echinarachnius* one may readily follow the changes leading to membrane production consequent on insemination: In the cortex beginning at the site of sperm entry droplets escaping push the membrane off. These droplets are discrete bodies that squeezed out of the cortex may cross the perivitelline space and reach the membrane before they go into solution. In other words, the *underlying process of membrane formation is a secretion or liquefaction of the cortex.* The result of this liquefaction is the wide perivitelline space and the diminution in the size of the egg. This process easily followed in *Echinarachnius* lends support to Loeb's suspicion that "the membrane formation is the result of a process of secretion of a liquid from the egg" (Loeb, 13, page 216). It also suggests the cortical secretion in the eggs of *Nereis* and *Platynereis.*

While we may say that in *Echinarachnius* certainly membrane lifting is not the cause but rather the result of activation, at least in sperm activation since the egg becomes immune to the entry of any other sperm before the membrane lifts (Just, '19*a*), yet membrane lifting is an important easily visible sign of complete activation.

3. The activated egg is an egg that has lost its fertilizin. The evidence of Lillie, Moore, and Just on this point working with two species of echinids and with *Nereis* shows very clearly that once activated, as shown by membrane production, the egg ceases to secrete fertilizin. Apparently, this loss is coincident with activation representing a neutralization of fertilizin with antifertilizin in the egg cortex.

We find these results, then, in the fully activated egg: (1) inability to respond to insemination, (2) the presence of a membrane widely separated from the vitellus which has arisen as the result of a cortical liquefaction, and (3) the absence of fertilizin. In the case of the sperm activated egg, the normal cleavage and development follow insemination; but in the butyric activated egg without hypertonic treatment, after a longer or shorter residence in sea-water cytolysis and death result.

Comparison with the over-exposed egg yields many dissimilarities.

1. The over-exposed egg is capable of insemination. Though it would appear Loeb failed to get such eggs to fertilize, the observations of Herbst, Moore, and Just seem to show that for a time at least after exposure these eggs will respond to insemination, albeit development is far from normal.

2. These eggs never form membranes on insemination.

In my judgment this failure to form a membrane after insemination is due to the loss of the membrane or membrane substance through too long exposure to the acid. I interpret the transparent jelly-like peripheral layer of these eggs to be not a swollen membrane but the cortex itself which swells as the result of the injurious action of the acid after loss of the membrane. This cortical change is the most striking characteristic of the over-exposed egg. In the activated egg, the cortex liquefies, pushes the membrane off and so brings about the formation of the perivitelline space; but in the over-exposed egg this cortex thickens.

3. The over-exposed egg, an egg still capable of fertilization, secretes fertilizin. On this point the studies of Moore admit no doubt. The over-exposed egg is not an activated egg.

As with the butyric acid activated egg without hypertonic

treatment the over-exposed egg cytolyzes. The rate of cytolysis, however, is so different that we must regard the process of different order in the two cases.

Over-exposure to butyric acid is cytolytic; hence, the egg rapidly dies, the cortical thickening is the beginning of cytolysis. In the case of the activated egg, cytolysis is entirely secondary; cytolysis takes place in spite of activation not because of it. Moreover, that which first cytolyzes in the over-exposed egg, namely the cortex, is absent in the activated egg because it has liquefied. Therefore, the cytolysis in the two eggs is qualitatively different.

For these reasons, therefore, we must conclude that *the over-exposed egg is a cytolyzing egg but that activation is not a cytolysis.*

But since according to Loeb all activation is caused by a "superficial" cytolysis we must examine further his widely accepted theory.

A. The Cytolysis Theory of Loeb.

In his book "Artificial Parthenogenesis and Fertilization," Loeb tells us: "The object of these experiments was the substitution of physico-chemical agencies for the mysterious complex 'living spermatozoön.'" "This book gives a survey of the methods by which the unfertilized egg can be caused to develop into an embryo and the conclusions which can be drawn concerning the mechanism by which the spermatozoön produces this effect. The theory which the author published in 1905 and 1906 that at least two factors are involved in this process, namely, one which brings about a change in the surface of the egg (the essential factor) and a second, corrective factor, seems to explain all the phenomena observed in the new territory and has proved a reliable guide." Thus Loeb despite his numerous other suggestions as to the cause of development quite definitely commits himself to the cytolysis theory that the egg either by the "mysterious complex living spermatozoön" or by the "substitution of physico-chemical agencies" develops through a change in its surface which he calls "superficial cytolysis." Against this theory of activation several potent objections may be raised.

The cytolysis theory is based on results obtained with over-exposed eggs. Loeb argues that because a toxic agent—like saponin, benzole, toluene, etc.—destroys the egg, its normal development or its artificial parthenogenesis is likewise a destructive (cytolytic) process solely because in many cases the toxic agent if allowed to act but a short time induces membrane formation; prolonged exposure being cytolytic, the shorter exposure which induces membrane formation must therefore be cytolytic. It would be tedious to cite the pages in which this argument appears. A single quotation will suffice. "We can therefore say briefly that *all hemolytic agencies effect the activation of the unfertilized egg and this activation consists in a cytolysis of the cortical layer of the eggs.*" The italics are Loeb's.

Moreover, Loeb practically tells us, as pointed out above that with *Arbacia* he employed over-exposed eggs and also that he used as a criterion for activation the rapidity with which the death changes (cytolysis) set in after treatment. The proper exposure for *Arbacia* eggs as first determined by Heilbrunn is much shorter than that which Loeb used and gives beautiful full membranes instead of the jelly-like film of the over-exposed egg. This over-exposure, therefore, simulates neither the cortical changes induced by the "complex 'living spermatozoön'" nor the artificial membrane formed through properly timed exposure.

The cytolysis theory, in the next place, is based on the superficial resemblance of cytolyzing eggs to cleaving eggs. Loeb continually speaks of "cleavage" when he means disintegration. Thus, page 76, he figures the "slow disintegration of the egg of the sea urchin at low temperature" which "can reach the eight cell stage." Butyric acid does not call forth cleavage in these urchin eggs. Herlant records much the same experience. Loeb also states that "centrosomes and two astrospheres are formed and the nucleus divides." Again, in the experience of others (cf. Herlant, also Just) the egg treated with butyric acid alone does not go beyond the monaster stage.

Likewise, on purely logical grounds the cytolysis theory should be rejected. Influenced in large measure no doubt by the interest then prevailing in the field of hemolysis, Loeb sought to bring the explanation of his work on "artificial parthenogenesis" in

line with these current researches. He therefore conceived "artificial parthenogenesis" as a process comparable to hemolysis and all hemolytic agents as artificial excitants of development. Since, therefore, fertilization is to be regarded as a sort of "artificial parthenogenesis" and according to Loeb formally explained by these experiments of his rather than by actual investigation of the fertilization process *per se,* the egg is fertilized by a sperm-borne lysin. Thus, on a mere assumption based on the similar action of toxic agents on blood cells and on ova he first explains "artificial parthenogenesis" and on this bases another assumption to explain fertilization. Now, of course the mammalian red blood corpuscle is *sensu stricto* not a cell: in the absence of a nucleus its metabolism is scarcely that of a normal cell, its anabolic activity must be limited, and its physical properties altered. While we lack convincing data for the estimation of the life of the erythrocyte we well know that it is on the way to destruction. Again, if we call to mind the various agents of hemolysis from the simplest, distilled water, to the most complex, snake venom or foreign sera, we must admit that they certainly do not normally occur in blood plasma. Hemolysis is artificial or pathological. Loeb compares the highly pathological process of hemolysis to the initiation of development of the animal egg —perhaps the most complex cell in the living world, richly endowed with synthetic power, a constructive mechanism of well organized hereditable qualities. On the one side we have the artificially induced disintegration of a piece of a cell, on the other the normal activation that initiates the development of the future metazoön. On purely logical grounds, therefore, the cytolysis theory is scarcely tenable.

If anything were wanting in the case against the cytolysis theory we could point out that for the sea-urchins of Loeb's experiments no single hemolytic agent acting alone induces development; they induce membrane formation or with longer exposure death. Eggs exposed to these so-called agents of development need for development (production of cell division and swimming larvæ) hypertonic treatment. It is the hypertonic seawater alone as Morgan long ago showed that induces cleavage. One would scarcely denominate hypertonic solution as hemolytic.

Finally, the cytolysis theory assumes that the cytolysis of the over-exposed egg is of the same nature as that of the egg which has had through a lower exposure a membrane formed. This is purely an arbitrary assumption. The over-exposed egg cytolyzes rapidly because it is injured; the egg with the membrane cytolyzes in spite of the membrane and at a slower rate. According to Herlant it is the failure to cleave that finally kills the membrane egg. The cytolysis in these two classes of eggs thus springs from different causes. Nor is this all. The thickening of the cortex of the over-exposed egg we may regard as the initial step in cytolysis—or, if you please, as the mark of the injury that hastens cytolysis. In the membrane egg, however, this cortex has broken down through a process of liquefaction or secretion which is the fundamental phenomenon in the cortical change of which membrane elevation is a sequel. Therefore, the cytolysis in these two kinds of eggs must differ not only in time but in the quality of substances that cytolyze.

For these capital reasons, then, added to the conclusion of the results on *Echinarachnius* any theory of activation that postulates the cause of the initiation of development as a superficial cytolysis is difficult of defence. The cytolysis theory of development has blocked the path of progress to an understanding of the fertilization problem.

B. The Rhythmical Changes of Cell Division.

Following successful insemination the egg divides. Cell division is thus a criterion of fertilization. Many of the phenomena, however, revealed by the egg subsequent to its insemination are to be regarded as belonging not to the fertilization-reaction but to the physiology of cell division. Among these we find rhythmical changes that parallel cell division. For example, we may cite those rhythmical changes in viscosity so carefully investigated by Heilbrunn. He correlates these changes with the appearance and growth of the mitotic spindle during the division cycle. Viscosity changes are, therefore, to be regarded as an index to cell division. They are manifestations of the rhythm of cell division and are in no wise peculiar to fertilization. Here too belong other changes in the egg which follow insemination,

such as: increased oxidation, increased permeability, varying susceptibility to heat, cold, ether, lack of oxygen, KCN, etc. They constitute indicia of cell division regardless of fertilization. An egg following insemination shows increase in viscosity, permeability, oxidation, and susceptibility to KCN. But these increases are not the "cause" of activation; they are the expression of the beginning of the rhythm of cell division.

If this position be tenable, if indeed these so-called indicia of cell division be simply the expression of cell division should we not expect to find that there are not such great changes in permeability, oxidation, or susceptibility to KCN in the case of an egg inseminated while in a stage of mitosis? Thus, in the sea urchin egg, in which maturation is complete and the nucleus at rest at the time of insemination, we should expect to find great physical and chemical changes due to the initiation of cell-division; but in an egg like that of the starfish inseminated during maturation we should not get such great changes. *And this indeed is the case.*

Loeb ('13) found, for example, in the sea urchin egg "that immediately after fertilization the egg consumed five to seven times as much oxygen as before fertilization." But he found conditions entirely different in the starfish egg: "No noticeable increase in the rate of oxidation is caused in this egg through the entrance of the spermatozoön. This is intelligible from the fact that those oxidations which lead to nuclear division were already going on in the eggs at the time the spermatozoön entered." In other words, insemination in the starfish egg does not initiate division and so does not increase the rate of oxidation.

R. S. Lillie has studied the permeability changes in fertilized eggs of *Arbacia* and *Echinarachnius,* both of which show the same kind of permeability changes. Thus, he found that *Arbacia* eggs take up water several times more rapidly after fertilization than before; so great is this difference as shown by the increased volume of the fertilized eggs that fertilized and unfertilized eggs in the same dish of hypotonic sea-water are easily separated. As would be expected, unfertilized and fertilized eggs differ in their response to hypertonic sea-water; the fertilized eggs lose water much more rapidly. *Echinarachnius* eggs behave similarly

but the eggs of Asterias show practically no difference before and after fertilization. These results indicate that permeability changes, like those of oxidation, are indicia of cell division.

Finally, Mathews has studied the effect of KCN on fertilized *Asterias* and *Arbacia* eggs. His findings on *Arbacia* confirm the earlier observations of Lyon on this egg. Mathews finds that the susceptibility of the starfish egg to KCN coincides with the development of the asters, while the period of immunity coincides with the retrogression of the asters. There is thus a rhythm of alternate increase and decrease of susceptibility to KCN which accompanies the rhythm of the mitotic process. The susceptibility to cyanide is, therefore, comparable to the rhythm of viscosity changes and to that of permeability.

With *Asterias* eggs, however, Mathews got unsatisfactory results. He could not discover in this egg the sharp periods of susceptibility to KCN discovered in the *Arbacia* egg. I venture the opinion that this result is due to the fact that in *Asterias* the maturation division is in process at the time of insemination. It would, therefore, be difficult if not impossible to obtain in the *Asterias* egg just after insemination a stage comparable to that in the *Arbacia* egg just after insemination. Indeed, at no time after insemination until the appearance of the cleavage asters could a fair comparison between these eggs be made.

In brief, these changes—of viscosity, oxidation, permeability, and susceptibility to KCN—are not changes incident to the fertilization-reaction *per se;* they are changes that are bound up with the problem of cell division. They are, therefore, in no wise peculiar to the fertilized egg. They cannot be regarded as explaining the activation of the egg.

C. The Fertilizin Theory of Lillie.

At present the best working hypothesis for the study of the fertilization-reaction is the fertilizin theory of Lillie. The fertilizin theory postulates that development is initiated through the ovogenous substance, fertilizin. The sperm activates the fertilizin and this reaction between sperm and fertilizin is the first step in the developmental processes in the egg. The activated fertilizin acts on both egg and sperm.

In the egg, as a result of the activation of fertilizin, cortical changes take place. These changes may be initiated through the mere attachment of the sperm to the egg membrane, as in *Nereis* and *Platynereis* where jelly secretion from the cortex takes place before the penetration of the sperm. In *Echinarachnius* cortical changes leading to the lifting of the membrane are induced by the sperm or by proper exposure to butyric acid.

Fertilizin likewise acts on the sperm; as Lillie puts it, the sperm itself needs to be fertilized. As a result of the union of the sperm with fertilizin, the sperm head is made to swell. If fertilizin be absent, as in immature eggs, in butyric acid membrane eggs (Moore) or in stale eggs, the sperm head does not swell. Spermatozoa may penetrate these three classes of eggs but they undergo no change nor do the eggs develop. We have thus two criteria of the sperm-fertilizin-egg reaction: the cortical changes in the egg and later the swelling of the sperm head. But even before this the reaction is complete. It is not a reversible destructive reaction in need of a "corrective factor" but a constructive, irreversible, *practically instantaneous reaction* setting in motion the whole train of events, with accompanying changes in oxidation, permeability, etc., leading to the cleavage of the egg.

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